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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

The purpose of this study is to understand the role of the p16 growth control axis in androgen dependent proliferation of prostate cancer cells. The p16 axis contains two tumor suupressors (p16Ink4a and pRB), cyclin D-dependent kinases, and transcription factor E2F. We hypothesized that functions of the p16 axis can influence androgen-dependence of prostate cancer cells. To test this hypothesis, we proposed to use controlled expression techniques to determine whether disruption of p16 axis function can lead to androgen-independence in human androgen-dependent prostate cancer cells (LNCaP) and, on the other hand, whether restoration of p16 axis function can restore androgen-dependence in androgen-independent prostate cancer cells (DU-145). During the second year of this study, we have established LNCaP derivative cell lines that can provide inducible expression of exogenously introduced proteins. In DU-145 cells, we have discovered that restoration of protein expression of pRB and the androgen receptor cause cell death. This finding reveals a novel functional relationship between pRb and the AR in prostate cancer biology.

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Introduction

The subject of this proposal is the role of the p16 growth control axis in androgen dependent proliferation of prostate cancer cells. The p16 axis contains two tumor suppressers (p16Ink4a and pRB), an important component of the cell cycle machinery (cyclin D-dependent kinases), and transcription factor E2F (regulators of gene expression). We hypothesized that functions of the p16 axis can influence androgen regulation of prostate cancer cells. To test this hypothesis, we proposed to use controlled expression techniques to determine whether disruption of p16 axis function can lead to androgen-independence in androgen-dependent prostate cancer cells and, on the other hand, whether restoration of p16 axis function can restore androgen-dependence in androgen-independent prostate cancer cells. Through this study, the connection between androgen regulation of prostate cancer cells and functions of a central cell growth control pathway will be revealed.

Body

The two approved aims in this project are discussed separately below.

<u>Aim 1. To determine whether deregulated expression of positive-acting cell cycle regulators can abrogate the dependence of LNCaP cells on steroid hormones in culture.</u>

The first objective of this aim is to establish LNCaP derivative cell lines that can express various cell cycle regulators in the p16 axis in a controlled manner. In the first annual report, we determined that the "tet-off" system was better suited for this cell line than the "tet-on" system.

During the second year of the grant, we attempted to establish LNCaP derivative cell lines after transfection with pUHD15-2 (encodes the tet-off tTA) and a drug resistance marker (Neo). We have used this approach successfully in establishing a number of inducible cell lines including the osteosarcoma cell lines U2OS and Saos-2 (Jiang et al., 1998; Jiang et al., 2000), and the prostate cancer cell line DU-145 (see below). Unfortunately however, out efforts to make such derivative cell lines from LNCaP cells met with significant difficulties due to the apparent degeneration of LNCaP cells after transient transfection. To circumvent this problem, we turned to retrovirus-mediated gene transfer technology. We obtained from Clontech a retrovirus vector that expresses tTA and Neo resistant gene from the same transcript through an internal ribosome entry site (IRES). We generated retrovirus stocks and infected LNCaP cells with this virus. After selection with G418, we established two clones (LNCaPtTA) that showed the ability to support tetracycline regulated expression of exogenous genes that are cloned under the tetracycline regulated promoter (pUHD10-3, (Gossen and Bujard, 1992)). In the experiment shown in Figure 1, one of the cell line (LNCaPtTA1-1) was transfected with pUHD10-3-L19 (here, L19 was used as a marker protein) plasmid in the presence and absence of tetracycline. Clearly, LNCaPtTA cells are able to provide tetracycline-controlled expression.

With LNCaPtTA cell lines, we have been trying to establish cell lines with inducible expression of E2F family members with
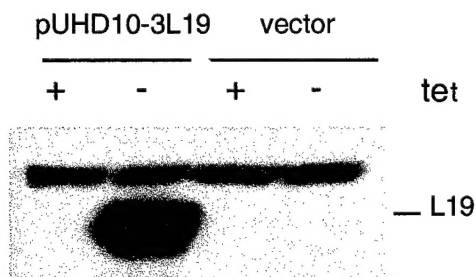

Figure 1. LNCaPtTA cells can support tetracycline regulated (tet-off) expression. Cells were transfected with the indicated plasmid (L19 is an HA-tagged protein used here only as a protein marker) in the presence and absence of tetracycline as indicated. Total cell extracts were Western blotted with anti-HA. A non-specific background band is present in all the lanes.

transfection of pUHD10-3-E2F1 and E2F2. We have been experiencing the same transfection-mediated cell degeneration problem as described above. We are currently trying to use retrovirus infection to introduce E2F genes under the control of the tetracycline responsive promoter. With the success in establishing LNCaPtTA cell lines with the retrovirus approach, we are confident that we will be able to establish inducible LNCaP cell lines for various cell cycle regulators as originally proposed such as cyclin D1, cyclin E, cyclin A, Cdk4, Cdk2, and E2F1 and E2F3. The effects of these positive cell cycle regulators on androgen dependence of LNCaP cells will then be determined.
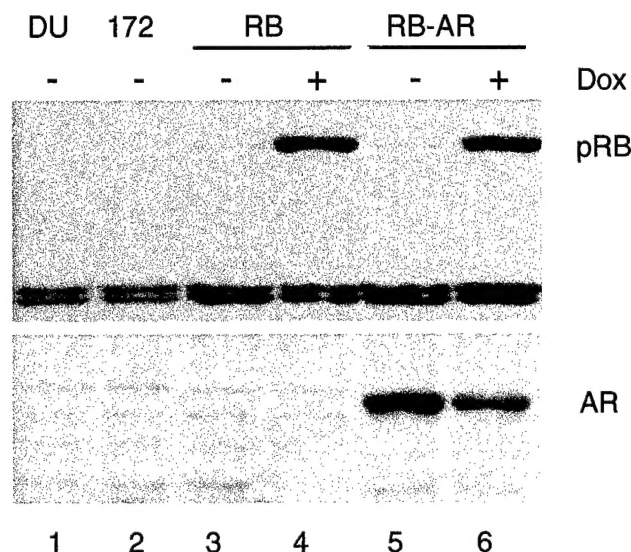

Figure 2. Expression of pRB and AR in DU-145 derivative cell lines. SDS-PAGE of total cell extracts, as indicated and see text for details, were Western blotted with the indicated antibodies.
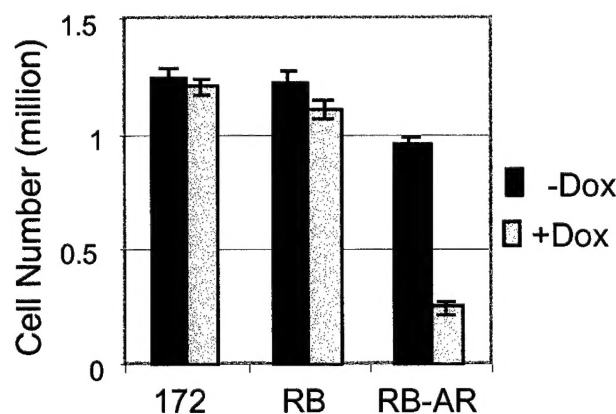

Figure 3. Inhibition of DU-145 cell proliferation by pRB and the AR. 400,000 cells were plated in the absence and presence of Dox and 60 hours later live cells (determined by Trypan Blue exclusion) were counted. Three sets of samples were used to obtain averages with standard deviations.

Aim 2. To determine whether correction of p16 axis function and androgen receptor expression can restore androgen dependence in DU-145 cells.

DU-145 prostate cancer cells do not express functional pRB and the androgen receptor (AR). Our first objective is to re-express functional forms of these two proteins. In the first annual report, we described that it was very difficult to establish AR-expressing derivative cell lines of DU-145 cells, whereas DU-145 cells with inducible expression of pRB were readily established with the "tet-on" system. We then tried to restore AR expression in the DU-RB cells by stably transfecting these cells with CMV-AR plasmid (constitutive expression of the AR) in the presence and absence of pRB induction. In this experiment, AR-expressing cell lines were readily established in the absence of pRB induction but not in the presence of pRB induction. Figure 2 shows the expression of pRB and the AR in representative cell lines. As expected, the parental DU-145 cells (lane 1) and the derivative that express rtTA (lane 2) all lack pRB and AR expression. The DU-RB cells can be induced to express pRB (+Dox, lane 4). However a very light band of pRB can also be detected in the absence of Dox (lane 3). We therefore suspect that very low levels of pRB expression (due to leaky control of the tet-on system) may have facilitated the establishment of AR-expressing derivative cell lines. The finding that AR-expressing cell lines were not identified in the presence of pRB induction suggested that the co-expression

of pRB and the AR in DU-145 cells may have inhibited cell proliferation and/or caused cell death.

We have carried out cell proliferation and cell death assays to determine the effects of pRB re-expression, in the absence and presence of AR expression, on DU-145 cells. Figure 3 shows the determination of cell proliferation by counting the numbers of live cells. From this experiment it is clear that the repression of pRB in the presence of the AR led to significant reduction in the number of live cells. In fact, the number of live cells after induction of pRB in DU-RB-AR cells actually decreased from the number of cells plated. The cell viability MTT assay showed the same result (see below). Significant amount of floating cells were also observed, suggesting that cell death was taking place as a result of pRB re-expression. Significantly, re-expression of pRB in the absence of AR expression did not have effects on DU-145 cell proliferation, which was consistent with previous reports (Bookstein et al., 1990).
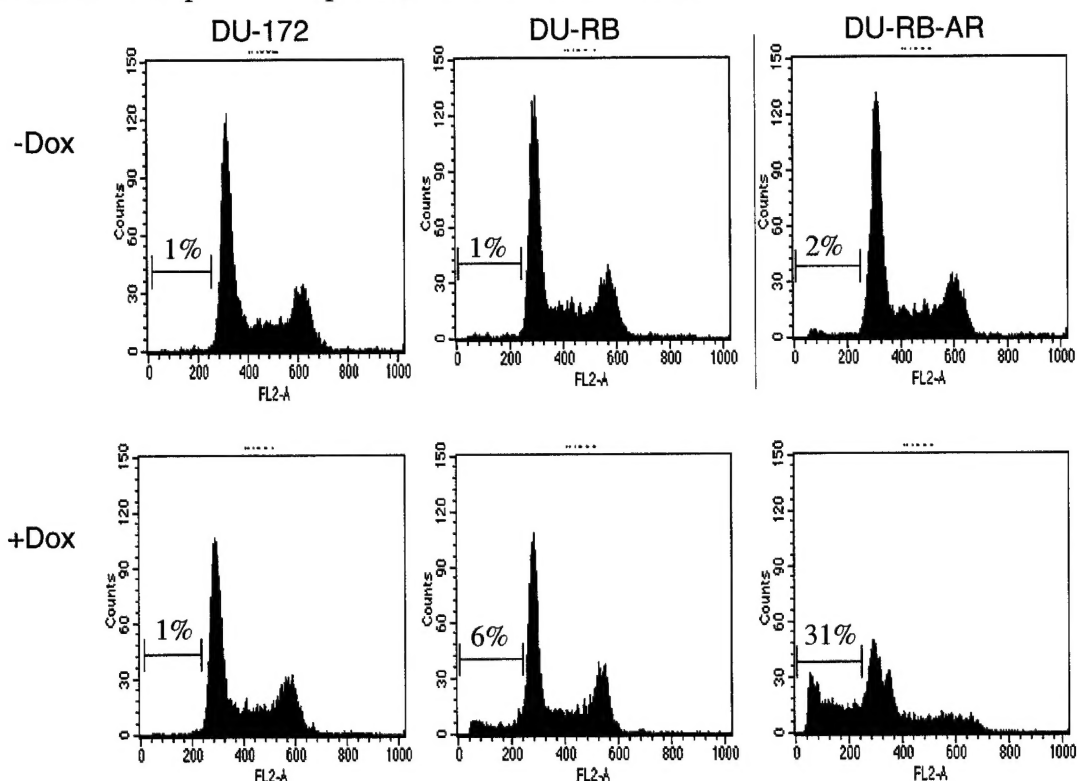

Figure 4. DNA fragmentation after induction of pRB in the presence of AR. The indicated DU-145 derivative cell lines were untreated, or treated with Dox, as indicated, for 60 hours and total cells harvested, stained with propidium iodide, and analyzed by flow cytometry. The percentages of cells with sub-G1 sized DNA (an indication of DNA fragmentation) are indicated.

To assay for the presence of apoptosis, we performed flow cytometry analysis to detect the presence of sub-G1 DNA fragments. As shown in Figure 4, induction of pRB in DU-RB-AR cells led to an increase in sub-G1 DNA fragments from 2% to 31%. In comparison, induction of pRB in DU-RB cells only increased sub-G1 DNA from 1% to 6%. The presence of apoptosis was also confirmed by the nuclear condensation morphology after staining the cells with acridine orange and ethidium bromide (data not shown). We therefore conclude that the co-expression of pRB and the AR in DU-145 cells induces apoptosis.
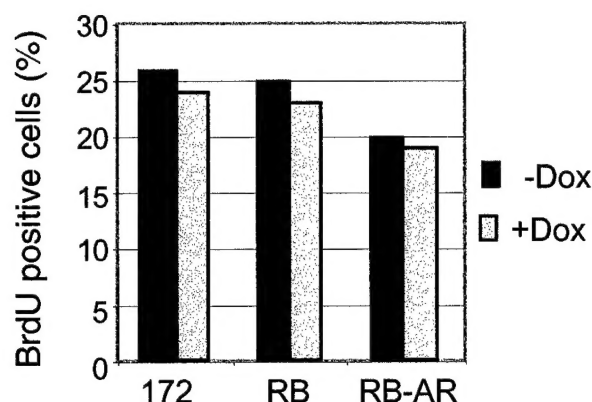
Figure 5. BrdU incorporation in a 3-hour pulse. The indicated DU-145 derivative cell lines were incubated in the presence and absence of Dox as indicated for 60 hours and labeled with BrdU for 3 hours. Incorporation of BrdU was determined by indirect immunofluorescence staining.

The best established function of pRB is negative regulation of G1-S phase transition in the cell cycle. To further determine whether this function of pRB was manifested after pRB induction in DU-145 cells, in the presence and absence of AR expression, we performed BrdU incorporation assay. As shown in Figure 5, induction of pRB in DU-RB-AR cells had no detectable effect on BrdU incorporation, suggesting that the G1 cell cycle arrest function of pRB is not the main activity in these cells.

Together, results in Figures 2 to 5 reveal a novel functional relationship between pRB and the AR. In the literature, pRB has been shown to confer DU-145 cells the ability to undergo apoptosis after irradiation (Bowen et al., 1998) and to be important for cell death when prostate cancer cells were deprived of matrix contact (Day et al., 1997). In general however, the AR is believed to promote survival of prostate epithelial cells. More recently, it was reported that the AR was required for activated MEKK1 to cause apoptosis in prostate cancer cells (Abreu-Martin et al., 1999). Physical interaction between pRB and the AR have been reported and this interaction was shown to stimulate AR transactivation activity in an androgen-independent manner (Lu and Danielsen, 1998; Yeh et al., 1998). The roles of these two proteins together in apoptosis of prostate cancer cells have not been known. Our findings reveal the existence of a cell killing pathway(s) that is dependent on the presence of both pRB and the AR.
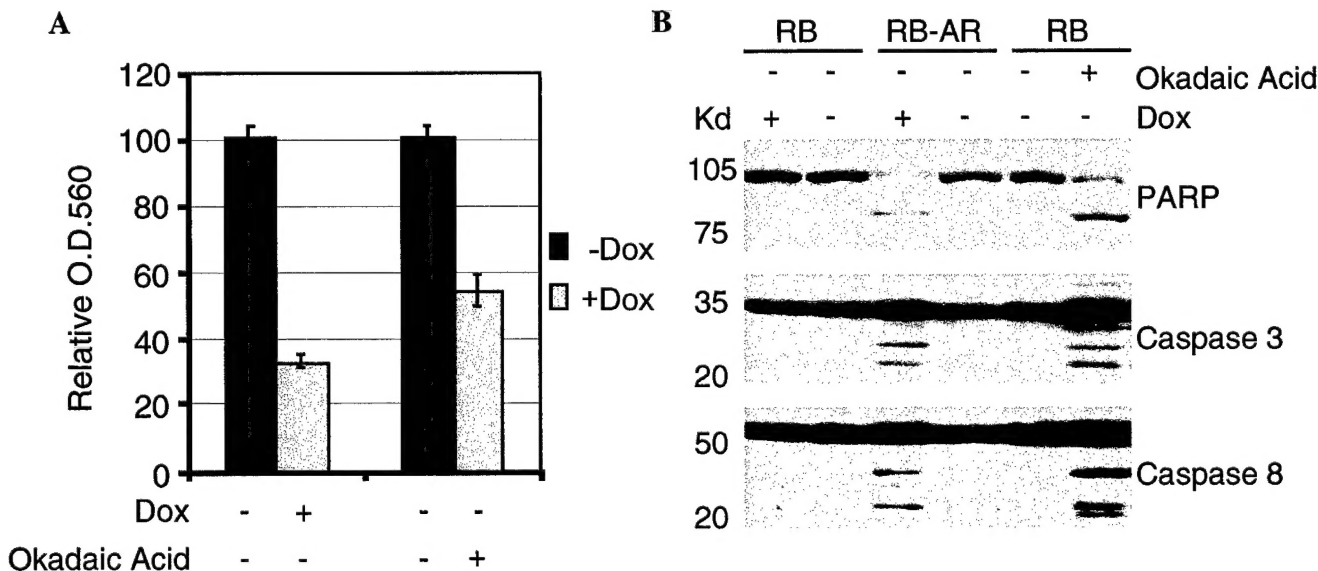

Figure 6. Cell death and caspase activation in response to pRB induction or okadaic acid treatment. A. DU-RB-AR cells were treated with Dox or okadaic acid (30 nM) as indicated for 40 hours. MTT assay was then performed and relative O.D. values are presented with standard deviations of three sets of samples. B. Identically treated cells were used for Western blotting with the indicated antibodies.

This suggests that the loss of pRB or AR function can confer survival advantage to the prostate epithelial cells and contribute to prostate cancer progression.

We have begun to investigate the biochemical and molecular mechanisms underlying this apoptotic response triggered by the co-expression of pRB and the AR in DU-145 cells. Treatment of DU-145 cells with okadaic acid is known to cause apoptosis through the activation of caspases (Bowen et al., 1998); we therefore used okadaic acid treatment in parallel to provide comparison. Proteolytic cleavage of the cellular protein PARP is a well established marker for the activation of the effector caspases 3. Activation of various caspases is also achieved through proteolytic cleavage of the procaspases. These cleavage events can be determined by Western blotting with the respective antibodies. We performed the experiment shown in Figure 6 to determine whether pRB and AR co-expression led to the activation of caspases compared with okadaic acid treatment. In panel A, we show that cell death mediated by the induction of pRB in DU-RB-AR cells is to a greater extent than those induced by okadaic acid treatment at a commonly used concentration (30 nM) as measured by the MTT cell viability assay. Panel B shows that proteolytic cleavages of PARP, the effector caspases 3, and the initiator caspase 8 are clearly taking place in DU-RB-AR cells after pRB induction to the same extent as those in okadaic acid treated DU-145 cells. These results establish that the combined expression of pRB and the AR in DU-145 prostate cancer cells results in caspase activation.

With the establishment of these DU-145 derivative cell lines and the identification of the apoptosis-inducing activity of pRB and AR co-expression, we are now in the position to investigate a novel functional relationship between the well-established tumor suppressor pRB and the AR in prostate cancer cells. Through these studies, we expect to provide new understanding on how survival is regulated in prostate cancer cells.

Key Research Accomplishments

- Established LNCaP derivative cell lines that provide tetracycline controlled expression.

- Established re-expression of the AR (constitutive) and pRB (inducible) in DU-145 cells.

- Discovered a novel functional relationship between pRB and the AR in the regulation of survival of prostate cancer cells.

Reportable Outcomes

A manuscript describing our finding that combined expression of pRB and the AR can stimulate prostate cancer cell death is in preparation.

Conclusions

One functional role of the tumor suppresser pRB in prostate cells may be to promote cell death in an AR-dependent manner. DU-RB-AR cells can be used to reveal the mechanisms of this novel functional relationship between pRB and the AR, which will significantly improve our understanding of the roles of pRB and the AR in the regulation of cell survival in prostate cancer.

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